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1 **Seed transmission of *Plectosphaerella cucumerina* of *Diplotaxis tenuifolia***

2

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9 **Abstract** Eight seed samples of wild rocket (*Diplotaxis tenuifolia*) obtained from commercial seed
10 lots used for sowing by farms severely affected by *Plectosphaerella cucumerina*, were assayed for
11 the presence of the pathogen. Isolations were carried out on subsamples of seeds (400) unwashed or
12 disinfected in 1% sodium hypochloride. The pathogenicity of the isolates of *P. cucumerina* obtained
13 was tested in two trials carried out on wild rocket. Four out of eight samples of rocket seeds were
14 contaminated by *P. cucumerina*. Among the eleven isolates of *P. cucumerina* obtained from 14,400
15 disinfected or not disinfected seeds tested, none was isolated from disinfected seeds. All isolates
16 were pathogenic on wild rocket. The results obtained indicate that rocket seeds are a potential
17 source of inoculum for *P. cucumerina*. The possibility of isolating the pathogen from seeds,
18 although from a low percent of them, supports the hypothesis that the rapid spread of this new
19 disease of rocket recently observed in Italy is due to the use of infected propagation material.
20 Measures for prevention and control of the disease are discussed.

21

22 **Key words** wild rocket, seed-borne pathogen, seed disinfection

23

24 **Introduction**

25

26 Wild rocket (*Diplotaxis tenuifolia*) is now widely cultivated and increasingly used in the
27 mediterranean cuisine both as a component of mixed salad and to decorate dishes. During spring
28 2012, symptoms of an unusual leaf spot disease were observed in several commercial greenhouses
29 near Salerno (southern Italy) and in northern Italy (near Bergamo) on plants of *Diplotaxis tenuifolia*
30 (cv. Selvatica). The first symptoms on leaves of affected plants consisted of small (1 mm) black-
31 brown spots of irregular shape, later coalescing into larger spots, 1 cm diameter. Spots were
32 surrounded by a yellow-gray halo, and were mostly located on the foliar limb, rib and petiole.
33 Affected leaves were often distorted, appearing hook-like. The disease was severe under 75-90%

34 RH, at air temperature of 20-26 °C, and caused severe production losses. Particularly, affected
35 tissues rotted quickly after packaging, during transit and commercialization of processed rocket
36 (Garibaldi *et al.*, 2012). The same pathogen is associated with root and collar rots of horticultural
37 crops in Italy (Matta and Garibaldi, 1980; Carlucci *et al.*, 2012) and has been very recently
38 observed on endive (Garibaldi *et al.*, 2013). On wild rocket (*D. tenuifolia*), the disease was not yet
39 reported in other countries.

40 Circumstantial evidence from surveys in the area interested by the disease suggested that the
41 sudden appearance of this disease was possibly due to the transmission of the pathogen by seeds.
42 The present study was undertaken to ascertain the extent of and the variation in occurrence of *P.*
43 *cucumerina* in rocket seeds.

44

45 **Materials and methods**

46

47 *Seed infection evaluation* Two seed samples of *Diplotaxis tenuifolia* were obtained from the
48 commercial farm where the disease was first observed (Salerno) and six seed lots were obtained from
49 commercial farms located in Lombardy, where the disease was later observed (Gilardi *et al.*, 2012).
50 A total of eight seed samples were assayed for the presence of *Plectosphaerella cucumerina* (Table
51 1).

52 Subsamples represented by 400 seeds were tested on 90 mm diameter Petri plates (10 seeds/plate)
53 containing potato dextrose agar (PDA) added with streptomycin sulphate at 25 mg L⁻¹, by following
54 the method described by Mathur and Kongsdal (2003). Isolations were carried out on seeds only
55 washed in distilled water (not disinfected) or disinfected by soaking for 1 min in 1 % sodium
56 hypochloride and dried. Plates were incubated at 12 h/day of fluorescent light at 22 °C for ten days.
57 Forty plates/trial were prepared. Each sample was checked at least twice. Seeds infected by *P.*
58 *cucumerina* were surrounded by a whitish-orange mycelium. The identification of the colonies of *P.*
59 *cucumerina* were confirmed by microscopic observation (Palm *et al.*, 1995) and by molecular
60 analysis (Garibaldi *et al.*, 2012).

61

62 *Isolates used and their preservation* The isolates obtained from seeds were coded as reported under
63 tables 3 - 5. Two strains of *P. cucumerina* from Salerno (southern Italy) (coded RS-CC1, GenBank
64 Accession No. AB469880) and PLC-27 from Bergamo (northern Italy) respectively, were used as
65 controls. The different strains were maintained on PDA at 8 °C.

66 *Production of inoculum and pathogenicity test* The different isolates of *P. cucumerina* were grown
67 in Petri plates on PDA added with 25 mg L⁻¹ of streptomycin sulphate, incubated at 12 h/day of

68 fluorescent light at 23 °C for 7 days. Spore suspensions were prepared from the single isolates
69 (Table 5). The concentration of spores was determined by hemacytometer and adjusted with
70 deionized water to 1×10^6 CFU (colony forming units) ml^{-1} .
71 Seeds of *Diplotaxis tenuifolia* cv. Selvatica (Suba), previously disinfected by soaking for 1 min in
72 1 % sodium hypochloride (disinfected) and washed in distilled water were sown in a steamed soil
73 mixture [with steamed mix soil of 50% Tecno2 (70% white peat and 30% clay) and 50% of Tiesse3
74 (60% white peat, 20% clay 20% perlite), Turco Silvestro terricci, Bastia d'Albenga, SV] in 2 L pots
75 and maintained at 25°C, with 12 hours/day of fluorescent light. Three replicates were used. Each
76 replicate consisted of 10-15 plants.
77 Thirty-day-old plants were artificially inoculated by spraying with a spore suspension (1×10^6 CFU
78 ml^{-1}) of the different isolates. As comparison, the isolates of *P. cucumerina* coded RS-CC1 and
79 PLC-27 obtained from wild rocket in Salerno and in Lombardy, respectively were used. Control
80 plants were sprayed with water.
81 Typical symptoms of *P. cucumerina* started to be visible 8 days after artificial inoculation. Plants
82 were checked for disease development and the percent of infected leaves was evaluated. The data
83 are expressed as percent of infected leaves 15 days after the artificial inoculation (Table 5). *P.*
84 *cucumerina* was consistently reisolated from the lesions. Data were statistically processed by means
85 of variance analysis ANOVA and Tukey test ($p < 0.05$).
86

87 **Results and discussion**

88

89 Four out of eight samples of wild rocket seeds, used for sowing in farms severely affected
90 by *P. cucumerina*, were contaminated by the pathogen (Tables 3 and 4) and eleven isolates were
91 obtained out of 7,200 not disinfected seeds. From disinfected seeds it was not possible to isolate any
92 strain of *P. cucumerina*.

93 Eleven isolates of *P. cucumerina* obtained from the different seed lots, were coded (Tables 3 and 4),
94 maintained in culture and tested in two trials for their pathogenicity on *D. tenuifolia*. The two trials
95 provided consistent results. All eleven isolates obtained from seeds were pathogenic on the cultivar
96 Selvatica (Table 5); inoculated plants showed typical symptoms. The virulence of the isolates
97 obtained from seeds was similar to that of isolates obtained in the field from infected plants of *D.*
98 *tenuifolia*.

99 The recent outbreak of *P. cucumerina* on wild rocket represents a potential threat to rocket
100 production in Italy. The disease has been detected on wild rocket, widely grown for processing.

101 Identifying the primary source of inoculum is of critical importance for effective disease
102 management.

103 This paper provides evidence that *P. cucumerina*, is frequently seed-transmitted (four
104 samples out of eight were contaminated), which suggests that seeds may be important in
105 disseminating the pathogen.

106 The results of this study do not provide information on the effects of *P. cucumerina* on the
107 quality and germination ability of rocket seeds. The results of this study indicate that rocket seeds
108 are a potential source of inoculum for development of *P. cucumerina*. The fast spreading of the
109 disease that occurred first in southern Italy in 2012, fastly moving in a few months to northern Italy
110 (Gilardi *et al.*, 2012) permits to hypothesize that the pathogen was introduced in Italy through
111 infected seeds.

112 Further research should be carried out to determine the epidemiological significance of seed-
113 borne inoculum as well as efficient methods to eliminate this threat to rocket production. The use of
114 *P. cucumerina*-free certified propagation material will become an essential qualification to
115 worldwide distribution of this crop. Seed dressing with registered and effective fungicides should
116 also represent one more option for disease management. Such treatments should also take into
117 consideration the possible contamination of rocket seeds, as already reported, by Fusarium wilt
118 agents (Garibaldi *et al.*, 2004). The fact that no isolates were obtained from disinfected seeds allows
119 to speculate that the pathogen is an external contaminant of seeds. In such a case, seed disinfection
120 should help reducing the dissemination of the pathogen. Beside the use of chemicals, also other
121 control methods should be exploited: a method based on the use of aerated steam, which proved
122 effective in the control of seed-borne diseases of cereals (Forsberg *et al.*, 2005) and of legumes will
123 be tested, as well as the use of biocontrol agents and natural products (Tinivella *et al.*, 2009).

124 Since the conventional pathogen detection techniques may lack the sensitivity required to
125 detect seed-borne pathogens, the detection threshold of *P. cucumerina* in rocket seeds could be
126 increased by using molecular techniques, such as PCR and RAPD as already shown in the case of
127 Fusarium wilt of basil (Chiocchetti *et al.*, 2001), lettuce (Pasquali *et al.*, 2007; Mbofung and Pryor,
128 2010) and other vegetables (Lievens *et al.*, 2012) and in the case of *Phoma valerianellae* in lamb's
129 lettuce seeds (Pellegrino *et al.*, 2010). Interestingly, it should be noticed that PCR and Real-Time
130 PCR methods have been already developed for the detection and quantification of *P. cucumerina*,
131 when used as biocontrol agent of potato cyst nematodes (*Globodera* spp.) (Atkins *et al.*, 2003).

132

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139

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Table 1 List of seed samples of *Diplotaxis tenuifolia* tested

Code/lot	Cultivar	Location of the farm	Seed Company
1A	Rucola selvatica	Salerno (NA)	Anseme (Cesena, FC)
2S	Rucola selvatica	Salerno (NA)	Suba (Longiano, FC)
71/CB	Winter	Bergamo (BG)	Orosem (Azzano S. Paolo, BG)
1387/2805PP	Charisma	Bergamo (BG)	Cora Seeds (Martorano, FO)
R 102033	Giove	Bergamo (BG)	T&T (Sant'Anna Di Chioggia, VE)
43/FRC-7	Extra	Bergamo (BG)	Franchi Sementi (Grassobbio, BG)
B101448	Venere	Bergamo (BG)	T&T (Sant'Anna Di Chioggia, VE)
31CM-1	Summer	Bergamo (BG)	Orosem (Azzano S. Paolo, BG)

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Table 2 List and layout of the trials carried out

Trial N.	Seed samples evaluated	Number of seeds evaluated, disinfected (D) and not disinfected (ND)	Date
1	1A; 2S	400 D /400 ND	13/06/2012
2	1A; 2S	400 D /400 ND	24/07/2012
3	1A; 2S	400 D /400 ND	11/10/2012
4	Winter, Charisma, Giove, Extra, Venere, Summer	400 D /400 ND	25-29/07/2012
5	Winter, Charisma, Giove, Extra, Venere, Summer	400 D /400 ND	21-24/09/2012

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203 **Table 3** Evaluation of the presence of *P. cucumerina* from seed samples of wild rocket obtained
204 from a farm at Salerno, the area of first detection of the disease

Trial N.	Seed samples evaluated	Number of <i>P. cucumerina</i> colonies (isolate code) detected out of 400 seeds tested/sample	
		Not disinfected (ND)	Disinfected (D)
1	2S	3 (17RS2-ND; 18RS2-ND; 19RS2-ND);	0
	1A	0	0
2	2S	2 (22RS2-ND; 15RS2-ND)	0
	1A	1(6RS1-ND)	0
3	2S	2 (3RS2-ND; 4RS2-ND)	0
	1A	0	0
Total number of <i>P. cucumerina</i> colonies out of 4,800	2S	7 (0.29%)	0 (0%)
	1A	1 (0.04%)	0 (0%)

205

206 **Table 4** Evaluation of the presence of *P. cucumerina* from seed samples of wild rocket from the
207 Lombardy area

208

Trial N.	Seed sample	Number of <i>P. cucumerina</i> colonies (isolate code) detected out of 400 seeds tested/sample	
		Not disinfected (ND)	Disinfected (D)
1	Summer Charisma,	0	0
	Extra, Venere,		
	Giove,	1(6G-ND)	0
2	Winter	1 (27W-ND)	0
	Summer Charisma,	0	0
	Extra, Venere,		
	Giove,	0	0
Total number of <i>P. cucumerina</i> colonies out of 9,600	Winter	1(3W-ND)	0
		3 (0.03%)	0 (0%)

209 **Table 5** Virulence of different isolates of *P. cucumerina* from infected seeds, expressed as
 210 percentage of infected leaves 15 days after the artificial inoculation
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Isolate code (from trial number)	Seed lot	Cv (Seed company)	% of infected leaves	
18 RS 2-ND (1)	2S	Selvatica (Suba)	40.0	bc ^y
19 RS 2-ND (1)	2S	Selvatica (Suba)	53.3	bc
20 RS 2-ND (1)	2S	Selvatica (Suba)	46.7	bc
22RS2-ND (2)	2S	Selvatica (Suba)	33.3	b
15RS2-ND (2)	2S	Selvatica (Suba)	63.3	c
6RS1-ND (2)	1A	Selvatica (Anseme)	43.3	bc
3RS2-ND (3)	2S	Selvatica (Suba)	56.7	bc
4RS2-ND (3)	2S	Selvatica (Suba)	33.3	b
6G-ND (4)	R 102033	Giove (T&T)	40.0	bc
27W-ND (4)	71/CB	Winter (Oroseme)	56.7	bc
3W-ND (5)	71/CB	Winter (Oroseme)	56.7	bc
RS-CC1 ^x	-	-	60.0	c
PLC-27 ^x	-	-	53.3	bc
Not inoculated control	-	-	0.0	a

212 ^x Control strains, RS-CC1 and PL-C27, obtained from infected leaves of wild rocket

213 ^y Means in the same column, followed by a common letter, do not differ significantly according to
 214 Tukey's test (P<0.05).

215